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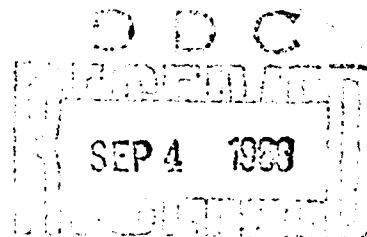
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COOPERATION OF INTRACELLULAR NADH-OXIDATION SYSTEMS

Following is a translation of an article by C. Wagenknecht and S. Rapoport of the Physiological-Chemical Institute at Humboldt University in Berlin, in Acta Biologica et Medica Germanica No 12, 1964, pages 542-551.

Summary

In model experiments the authors examined cross-connections with the main pathway of the respiratory chain in mitochondria. They ascertained reactions between the soluble diaphorases of the cytoplasm and the mitochondria. At the same time, they traced the possibility of reaction between microsomes and mitochondria in the model and examined it as to its mechanism of reaction. The electron transfer of this respiratory cooperation is not based on an absorption or diffusion process. As far as the cross-connections of the respiratory pathways in the cell are concerned, the ferric flavin enzymes of the mitochondria play a significant role as a center of cooperation. The importance of the cooperative ways in the cell is discussed.

As it has been known for a long time, the respiration of cells may be stopped almost completely through cyanide and carbon monoxide which have a selective effect on cytochrome oxydase (1 - 3). As a result, cytochrome oxydase, which is localized in the mitochondria, represents the final stretch of oxidation of the entire cell. Enzymes which transfer hydrogen but fail to react directly with oxygen, may be found in large numbers and great capacity outside of the mitochondria. The normal enzyme capacity of a rat's liver shows, for example, that of the total NADH dehydration capacity only 40% are localized in the mitochondria, 50% in the microsome fraction [See Note], and 10% in the soluble phase. It may therefore be assumed that cross

connections do exist between the final stretch of oxidation and the mitochondria. This work uses model experiments to examine the cooperation of respiratory enzymes in the cell.

(/Note:7 Ergastoplasmic membranes were not separated from the ribosomes in these experiments. Instead, the mixture -- also called microsomes -- was used.)

Material and Method

The cell matter used -- mitochondria, heart enzyme, microsomes, and hyaloplasm, as well as NU and RU (/See Note/ -- was represented as described in earlier publications (4 - 7).

(/Note:7 The following abbreviations were used: HE = heart enzyme; NU = static erythrocyte fluid; RU = static reticulocyte fluid; NADH = nicotine acid amino-in-dinucleotide, reduced.)

A commercial preparation of NADH was used as the substratum. The NADH oxydase activity was measured through extinction decrease at 340 nm. The measuring took place in a 0.03 M phosphate buffer, pH 7.4; the final volume amounted to 2.8 ml. Generally, the following final concentration per deposit was maintained: NADH = 200 g; HE = 30 g albumen; microsomes = 90 g albumen; NU + RU = 130 g albumen.

If highly viscous media were used, the reaction was observed following thorough mixing by means of a glass rod which lasted for 30 seconds without creating air bubbles.

All units of activity are expressed in uval/min/ml enzyme solution.

Results

1. Diaphorase Reaction in the Soluble Phase and the Mitochondria

Table 1 shows the influence of erythrocyte NADH diaphorase, which is contained in NU, on the NADH oxydase system of the heart enzyme. Static erythrocyte liquid (NU) was added as a diaphorase to a heart sarcosome preparation. NU itself showed no NADH decrease. The oxidation of NADH through heart sarcosomes was increased by a factor of 2.9 with the aid of added NU.

Table 1

NADH Oxidation Increase in the Mitochondria
Through Erythrocyte Diaphorases

a) Ansatz	NADH-Oxydation μMol/min	b) Effekt c) HE + Diaphorase HE	d) Hemmung %
HE	0.515		
HE + NO	1.503	3.9	
HE + RU	0.475		80
(HE + RU) + NO	0.555		90

Legend: a) Additive; b) Effect; c) Diaphorase; d) Retardation.

Static reticulocyte liquid, which contains a similar amount of diaphorase activity (5), did not effect increased reaction between NADH and oxygen. The reason for this lies no doubt in the fact that RU contains the retarding elements of mitochondrial ferric flavin enzymes. Since the RU retarding elements check the reaction between NADH cytochrome c-reductase and the cytochrome c, the absence of an increase in NADH oxidation through NU leads to the assumption that the reaction passes through the blocked factor of NADH cytochrome c-reductase of the mitochondria.

2. Reaction in the Mitochondria and Microsomes

Heart enzyme, porcupine microsomes (8), and rat liver mitochondria and microsomes were used in these experiments. Table 2 indicates that the addition of microsomes can increase NADH oxidation in the mitochondria by a factor of 3.6. In controlling experiments, the microsomes alone did not effect a NADH decrease, that is, no activity was observed in the NADH oxydase test of the microsomes (8). Since washed cell particles were used, we are justified in assuming that after mitochondria were added, the microsomal NADH cytochrome reductase found a connection with the final stretch of oxidation in the mitochondria. The experiment indicates that microsomes act as soluble diaphorases. Pre-incubation of heart mitochondria with RU greatly retarded NADH oxydase. Microsomes, which were added subsequently, failed to effect an increase of NADH oxydase system.

An earlier study (9) demonstrated that RU has no influence on microsome cytochrome reduction. RU blocks the ferric flavin of the mitochondria. Consequently,

Table 2

The Increase of NADH Oxidation in Heart Sarcosomes
Through Porcupine Microsomes

a) Ansatz	NADH-Oxydation b) $\mu\text{mol/min}$	Effekt c)	Hemmung d) %
HE	1.59	—	—
HE + Mkr.	5.72	3.6	—
HE + RU	0.16	—	90
HE (+ RU) + Mkr.	0.30	—	90

[Legend]: a) Additive; b) Oxidation; c) Effect; d) Retardation.

cooperation of the two cell particles may be located either at the point of RU action, or just below it.

The capacity of the oxidation path from cytochrome c to cytochrome oxydase, which is larger than that of the mitochondrial NADH cytochrome c-reductase, may be titrated by adding an overdose of microsomes. In the experiment, which is recorded in figure 1, the concentration of mitochondria was kept constant, and the maximum capacity of the oxidation pathway from cytochrome c to cytochrome oxydase was determined by adding greater amounts of microsomes. The relative oxidation increase does not depend on the amount of mitochondria, provided that there is a surplus of microsomes. This experiment is illustrated in figure 2. Different amounts of a mitochondria suspension were added to a great surplus of microsomes (the concentration was saturated by a factor of 10). Microsome values follow a straight line, just as those of the mitochondria. Accordingly, there was also a constant increase factor.

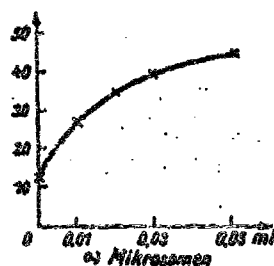


Fig. 1 Titration of the oxidation pathway from cytochrome c to the cytochrome oxydase

of heart sarcosomes through porcupine microsomes. NADH oxidation of an HE preparation was tested following the addition of various amounts of porcupine microsomes (HE constant). The ordinate marks the activity as an extinction change at 340 nm; the abscissa shows various amounts of microsomes in ml primary solution. Measurements took place in 0.03 phosphate buffer pH 7.4, end volume 2.8 ml.

[Legend]: a) Microsomes.

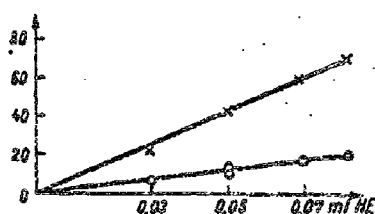


Fig. 2 Constant increase of NADH oxydase of heart sarcosomes through porcupine microsomes.

NADH oxydase of heart sarcosomes was tested for proportions with a surplus of porcupine microsomes (x) and without the addition of microsomes (o). Ordinate: activity as a decrease in extinction at 340 nm; abscissa: various amounts of a diluted heart preparation.

3. The Mechanism of Reaction

There are three possibilities for the mechanism of reaction in the two enzyme systems:

- a) The absorption of microsomes by the mitochondria.
- b) A diffusion of the microsomes and the mitochondria, related to a transfer of electrons.
- c) A distant action, the transfer of electrons by means of matter with a low molecular content.

First of all, tests were conducted to determine whether or not the cooperation effect between mitochondria

and microsomes was retained after the mitochondria (HE) were separated through centrifuging (12,000 g). Positive results would have indicated an absorption of microsomes. The last line in table 3 shows that mitochondria, re-suspended from the activated mitochondria-microsome accumulation, have a lower activity than the initial non-activated suspension. Both after and prior to centrifuging, additional microsomes increased the activity by a factor of three. Consequently, an absorption of microsomes by the mitochondria does not apply.

Table 3

The Influence of Centrifuging on the Cooperation
Effect Between Heart Sarcosomes and
Porcupine Microsomes

	1 ohne Mikrosomen	2 mit Mikrosomen	3. Aktivitäts- Koeffizient
HE vor Zentr. 4)	13	40	3.1
HE nach Zentr. 5)	5	17	3.4
HE + Mikr. nach Zentr. 6)	7	22	3.1

Legend: 1) Without microsomes; 2) With
microsomes; 3) Activity coefficient; 4)
Prior to centrifuging; 5) After centrifuging;
6) Microsomes after centrifuging.

The role of diffusion was tested in experiments where the viscosity of the medium was varied. Cane sugar was added in varying amounts to increase viscosity. Table 4 shows the NADH oxydase activity of the mitochondria and microsomes in media of varying viscosity. In viewing the rate of reaction in sediments without microsomes, we find a reciprocal relationship between the rate of reaction and viscosity. Starting with the reaction in 0.03 M phosphate buffer pH 7.4, the activity was calculated in accordance with the expected influence of viscosity on the diffusion rate of materials, using the formula activity = activity in PO_4 -buffer

$\sqrt{\text{Viscosity}}$. The figures are indicated in table 4 in parentheses. A similar dependence is found in sediments containing microsomes. The increase coefficient remains constant. These results lead to the conclusion

that the transfer mechanism between microsomes and mitochondria does not have an additional retarding effect on diffusion.

Table 4
Cooperation Between Heart Sarcosomes and
Porcupine Microsomes in Viscous Media

	4. Viskosität	1. HE	2. HE + Mikrosomen	3. Aktivitäts-Koeffizient 2/1
0,03M Phosphat, pH 7,4	1	19 (19)	59 (59)	3.1
in 40% Sucrose	5	10 (9)	40 (27)	4.0
in 50% Sucrose	12	7 (6)	20 (18)	2.9
in 60% Sucrose	40	3 (3)	10 (10)	3.3

Legend: 2) HE + Microsomes; 3) As in Table 3; 4) Viscosity; 5) Phosphate.

The mitochondria and microsomes were washed three times in an isotone salt solution with the attempt of removing matter with a low molecular content. This process did not reduce the increase in activity. Furthermore, various buffers were examined. It was determined that similar results are obtained in phosphate, tris-, and tra-buffers.

Discussion

This study, as well as model experiments described earlier (4), lead to the conclusion NADH oxidation of the cell may well take the course shown in figure 3. In that case, the final path of oxidation would always lead through the mitochondria and their cytochrome oxydase. The diagram shown stresses the microsome cytochrome and the iron in the NADH cytochrome c-reductase as key points in the cooperation of NADH oxidation.

This outline ignores the extra-mitochondrial NADH pathway which was described in the case of a locust muscle (10 - 15). According to these experiments, hydrogen of the extra-mitochondrial NADH cannot be directly oxidated from the mitochondria. It has been proved instead, that hydrogen is flooded into the mitochondria through the substratum α -glycerine phosphate.

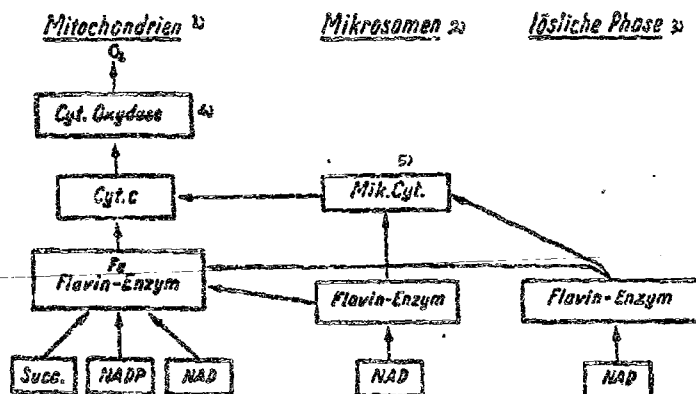


Fig. 3 Cooperation of NADH Oxidation

Legend: 1) Mitochondria; 2) Microsomes;
 3) Soluble phase; 4) Cytochrome oxidase; 5)
 Microsome cytochrome.

Another mechanism (16 - 18) is based on the theory that NADH does not permeate the mitochondria membrane. According to these findings, NADH and aceto-acetic acid at the surface of the mitochondria may react to β -oxybutric acid. The enzyme acting as the catalyst in this reaction, is then in a position to fill the mitochondria with hydrogen through an oxidation reaction. Only a catalytic amount of acetoacetic acid is necessary to produce such a reaction.

Earlier studies proved the existence of independent soluble NADH diaphorases in static haemolysate (5), as well as in the hyaloplasm of the liver, kidney, and heart tissue (4). Their possible biological importance has been discussed as a partial system of oxidation pathways in extra-mitochondrial NADH. On one hand, the NADH of the soluble phase -- as indicated by the results of this study -- might be oxidated through NADH diaphorase and the mitochondria. A direct electron transfer from the soluble diaphorase to the ferric mitochondrial flavin enzyme may be an ultra-mechanism of the reaction. Oxidation through the flavin enzyme of the ergastoplasmic membranes is another possibility. The electron transfer would continue from this system to the mitochondria. It should not be assumed that the DT-diaphorase isolated by Ernster (19 - 21) is identical with the diaphorase in the model system. The essential differences of these enzymes lie in the fact that the DT-diaphorase shows

no reaction with cytochrome b_5 , and is supposed to serve as a natural electron acceptor co-enzyme Q.

The oxidation of NADH through microsomes might find a connection to the main respiratory chain in direct reaction with the mitochondria. So far, a direct cooperation between microsomes and mitochondria in vivo has not been found yet. However, on the basis of the above model experiments, a direct reaction between microsomes and mitochondria seems possible. In considering an electron transfer between the particles, we should bear the fact in mind that a direct reaction between the microsome-cytochrome and cytochrome c is very rapid (as shown by Strittmatter and Velick 22) and probably rests on a simple chemical reaction. It would be difficult to imagine this rapid reaction as a mechanism of reaction since both cytochromes are particle bound.

These model experiments point to the possibility of an electron transfer between microsomes and mitochondria, even though the problem of the ultra-mechanism has not been solved as yet. In this case, the ferric components of the mitochondrial flavin enzyme are bound to participate in the mechanism of transfer, since RU, the inhibitor of ferric flavin enzyme of the mitochondria, retards cooperation.

The results of the model experiments lead to the conclusion that the reaction mechanism of electron transfer rests neither on the absorption of microsomes by the mitochondria, nor on the diffusion of particles and substances with a low molecular content. The following deliberation supports these results:

The reaction mixture contained 1.3×10^{-3} g albumen/ml mitochondria and 3.0×10^{-5} g albumen/ml microsomes as final concentration. The microsomes used was the mixture of ribosomes and ergastoplasmic membrane, obtained after separating the homogenous mixture. In order to determine the number of particles and the average distance between them, the average values shown in table 5 were used. Two methods were employed to calculate the number of particles. First, they were determined from the albumen concentration, divided by the molecular weight, and multiplied by the Loschmidt figure. Secondly, the weight of particles was calculated from the diameter and density in order to divide the concentration by the individual weight and to obtain the number of particles per ml. The figures obtained were 5.10^{12} /ml of microsome particles, and 5.10^8 /ml of mitochondria. For the calculation of average distances between particles, they may be regarded as points and their volume

may be disregarded. A regular statistical distribution of particles is used as the basis of the calculation. Consequently, the distance between microsomes is 0.6μ , and between mitochondria 12μ .

Table 5
Assumed Average Values for Microsomes
and Mitochondria

	¹⁾ Mikrosomen	²⁾ Mitochondrien
3) Durchmesser	20 nm	50 nm
4) Dichte	1.15	1.25
MG	10^7	10^{10}

[Legend]: 1) Microsomes; 2) Mitochondria;
3) Diameter; 4) Density.

These distances enable us now to calculate the diffusion time required to pass from one particle to another using Einstein's equation of the diffusion constant

$D = \frac{RT}{N_6 \pi n r}$. Accordingly, the diffusion constant of microsomes is $2.2 \times 10^{-9} \text{ cm}^2/\text{sec}$. Using these figures as a basis, we are now in a position to answer the question whether or not diffusion plays a role in the transfer of electrons. The assumed diffusion path of microsome particles would correspond to an average diffusion time of 1.7 seconds. The average distance between microsomes and mitochondria is 3 μ , which would correspond to a diffusion time of 42 seconds. In relation to oxidation, this would represent an extremely slow process. Since the mitochondria decompose approximately 10^7 NADH particles per minute, the diffusion rate of the particles is too slow by a factor of 6. This calculation clearly indicates that an electron transfer from the microsomes to the mitochondria cannot possibly occur by means of a diffusion process.

This becomes even less likely if we consider the co-operation experiment in a viscous medium. This experiment allows for the possibility of diffusion as a mechanism of transfer only if the transmitting agent represents a substance with a very low molecular content. A substance meeting this requirement did not appear in the model system. Neither could it have settled on the particles,

because repeated rinsing did not influence the cooperation effect. A substance with a low molecular content would have been washed away. The possibility of absorption of the microsomes by the mitochondria must also be ruled out. In the case of absorption, it should be possible to centrifuge the aggregated particles away. After the centrifugal process, however, the mitochondria may be activated to a similar degree as before.

Our process of elimination leaves two possibilities for the mechanism of transfer:

1. The transfer of electrons may occur through radicals which originate in water (O_2H and OH) and play the role of transmitting agent in the form of a chain reaction.

2. The principle of a semi-conductor or condensor may be considered as a possible transfer mechanism of electrons. In that case, the transfer of electrons from microsomes to mitochondria would take place in the form of a chain reaction, similar to that found in a semi-conductor. This theory is supported by the fact that the activity increase of the mitochondria always stands in a direct ratio to the sum of surface microsomes and mitochondria. Below the maximum activity, this ratio always shows the same quotient (Table 6). This relationship is similar to the ratio in a condensor where the capacity also depends on the surface.

Table 6

Dependence of the Cooperation Effect on the Activity Increase and Surface of Particles

1	2	3	4	5
Mikrosomen-Zusatz ml	Aktivität	Aktivitätssteigerung (gesamte Akt./Mit. Akt.)	Summe der Oberfläche cm ²	Effekt 3/4
-	13	-	4	-
0.01	24	11	13	0.8
0.02	27	14	18	0.8
0.03	33	20	25	0.8
0.05	46	33	49	0.7

[Legend]: 1) Microsomes added ml; 2) Activity; 3) Activity increase (Total activity per mitochondria activity; 4) Sum of surface cm²; 5) Effect 3/4.

The employment of radical catchers and the study of temperature and pH influences on the cooperation between mitochondria and microsomes might give us more information on this matter.

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